Mechanistic Studies on Lysine 2,3-Aminomutase: Carbon-13–Deuterium Crossover Experiments

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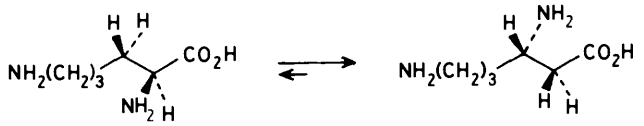
A mixture of L-[3,3-$^2H_2$]lysine plus L-[2-^{13}C]lysine is converted by purified lysine 2,3-aminomutase into β-lysine. The di-*N*-phthaloyl methyl ester derivative of the resultant β-lysine shows a geminally deuterium-coupled enriched ^{13}C n.m.r. signal indicating intermolecular transfer of the migrating 3-*pro-R* hydrogen of the α-lysine. L-[3-^{13}C]Lysine has been synthesized. A mixture of L-[3,3-$^2H_2$]lysine plus L-[3-^{13}C]lysine is similarly converted into β-lysine. Its di-*N*-phthaloyl methyl ester derivative shows a β-shifted enriched ^{13}C n.m.r. signal for C-3, but no geminally coupled enriched C-3 signal. The latter experiment rules out any intermolecular exchange of the non-migrating 3-*pro-S* hydrogen atom of α-lysine. Various mechanistic possibilities for the intermolecular hydrogen transfer process are discussed.

We recently reported the results of some mechanistic studies on lysine 2,3-aminomutase isolated from *Clostridium subterminale* strain SB4.[1] These studies concluded, *inter alia*, that, in the reversible interconversion of α-lysine (**1**) and β-lysine (**2**), the C-3



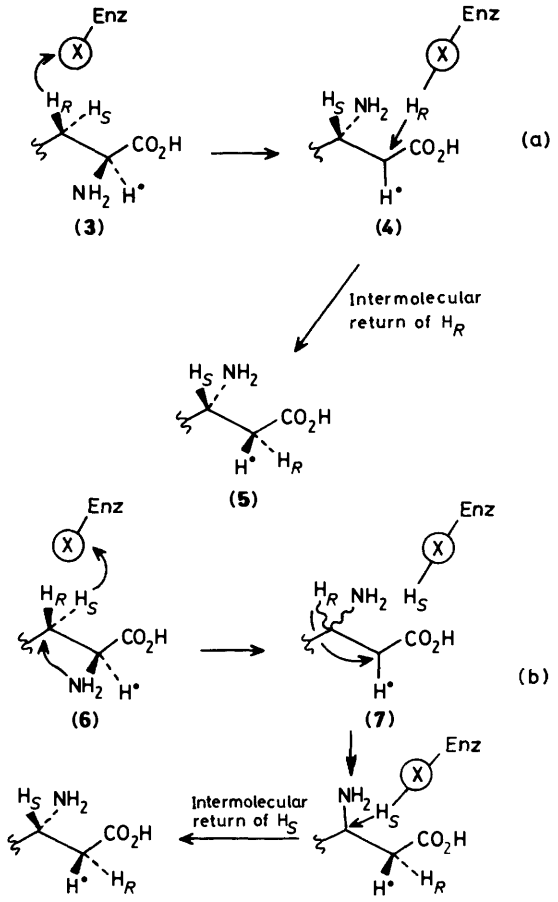
(**1**) (**2**)

to C-2 hydrogen migration which occurs concomitantly with the C-2 to C-3 *intramolecular* amino group transfer[2,3] takes place *intermolecularly* (Scheme 1a). This conclusion was based on mass spectrometric examination of the β-lysines derived from lysine 2,3-aminomutase transformation of mixtures of L-[3,3-$^2H_2$]lysine with L-[4,4,5,5-$^2H_4$]lysine or L-[U-^{13}C]lysine. The mass spectra revealed crossover of deuterium from the L-[3,3-$^2H_2$]lysine to the heavier co-incubated lysine. Since an alternative scenario (Scheme 1b) also consistent with the mass spectrometric results is conceivable in principle (albeit less likely), in which the hydrogen (deuterium) atom remaining at C-3 of β-lysine undergoes intermolecular exchange, we sought further evidence delineating the nature of this intermolecular hydrogen exchange. We now report the results of crossover experiments between L-[3,3-$^2H_2$]lysine and L-[2-^{13}C]lysine or L-[3-^{13}C]lysine, which support the conclusion that only the *migrating* (3-*pro-R*) hydrogen of α-lysine is involved in intermolecular exchange.

Results

L-[2-^{13}C]Lysine was obtained commercially and mixed with an equal amount of L-[3,3-$^2H_2$]lysine. The mixture was then incubated with purified lysine 2,3-aminomutase,[1] following the conversion by using a newly developed h.p.l.c. technique.[4] The reaction was allowed to proceed until *ca.* 60—65% of the α-lysine had been converted into β-lysine.† The resultant mixture of α- and β-lysines was converted into the di-*N*-phthaloyl methyl ester derivatives, (**10**) and (**11**), which were purified by preparative h.p.l.c.

The ^{13}C n.m.r. spectrum of the β-lysine derivative (**11**) showed, in particular, an intense signal at δ 36.65 accompanied by a higher-field triplet centred at δ 36.38 (*J* 20.8 Hz), shown in expanded form in Figure 1. The total integral of the upfield-shifted triplet, due to 2-^{13}C geminally coupled to deuterium,[6] was *ca.* one-half that of the δ 36.65 singlet. Thus *ca.* one-third of the ^{13}C-enriched β-lysine was formed with deuterium at C-2.‡



Scheme 1. Hypothetical mechanisms for intermolecular hydrogen transfer by lysine 2,3-aminomutase: (a) mechanism resulting in intermolecular exchange of the 3-*pro-R* hydrogen of α-lysine; (b) mechanism resulting in intermolecular exchange of the 3-*pro-S* hydrogen of α-lysine

† At equilibrium, at least with unlabelled substrates, *K* equiv. = 6.3, *i.e.* 86% of the α-lysine converted to β-lysine.[5]

‡ This estimate is not corrected for possible differences in nuclear Overhauser enhancement of the C-2 signal.

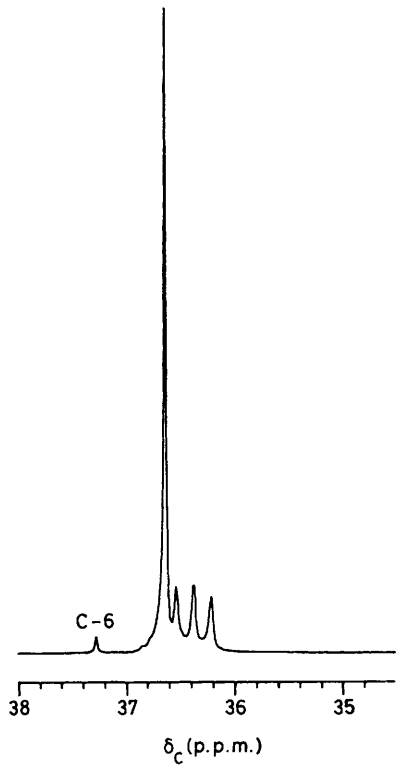
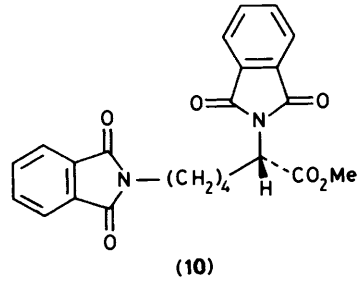
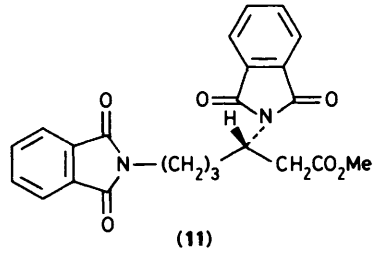


Figure 1. Expanded portion of the 125.76 MHz ^{13}C n.m.r. spectrum ($CDCl_3$ solution) of β-lysine di-*N*-phthaloyl methyl ester derivative (**11**) obtained after incubation of a mixture of L-[3,3-$^2H_2$]lysine + L-[2-^{13}C]lysine with lysine 2,3-aminomutase. The figure shows signals of C-6 (δ 37.30) and the uncoupled (δ 36.65) and deuterium-coupled (δ 36.38, *J* 20.8 Hz) signals of C-2. Conditions: sweep width 29 411 Hz, repetition rate 0.557 s, relaxation delay, zero, peak width 4.0 μs, line broadening 1 Hz, 1.795 Hz/pt; 1 405 scans



(10)



(11)

The data are roughly consistent with a completely intermolecular transfer of deuterium from C-3 of α-lysine to C-2 of β-lysine, if it is assumed that *ca.* two thirds as much [3,3-$^2H_2$]lysine as [2-^{13}C]lysine was converted into β-lysine. Considering the approach to equilibrium in the transformation which would, to some extent, have overcome the isotope effect ($K_H/K_D \simeq 3$) seen previously[1] for C-3 dideuteriation, this assumption seems reasonable. It is supported by the C-4 ^{13}C n.m.r. signals: whereas unlabelled (**11**) showed a single peak for this carbon at δ 29.72, the product obtained from the above experiment showed two peaks (δ 29.68 and 29.58; intensity ratio 1.57), the less intense upfield peak being that of the C-4 resonance which had been β-shifted[7] by the neighbouring C-3 deuteron in (**11**) derived from [3,3-$^2H_2$]lysine. (This shift of the C-4 resonance will occur whether or not the C-3 deuteron is involved in intermolecular exchange).

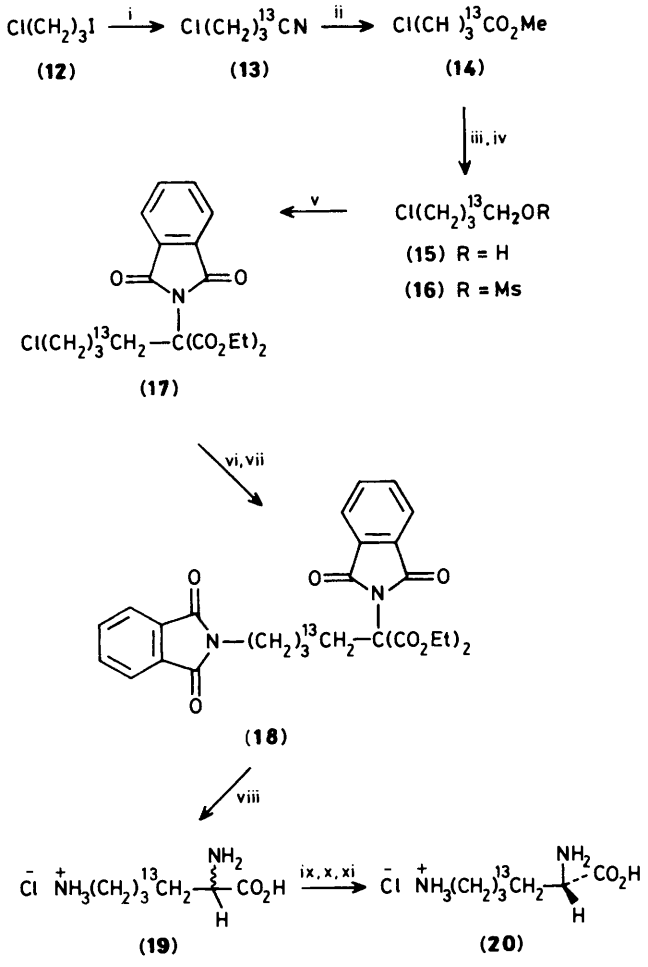
The spectrum also showed single lines for C-5 (δ 25.51) and C-6 (δ 36.88), but the signals for C-3 were complex, having apparently two main lines (δ 47.74 and 47.44) due, respectively, to unshifted and β-shifted C-3 in the [2-^{13}C]lysine-derived product with and without, respectively, intermolecularly transferred deuterium at C-2. However, 3-^{13}C signals from directly deuterium-bonded carbon (product derived from [3,3-$^2H_2$]-lysine) could not be clearly seen due to the very weak signal intensity.

Thus, the above n.m.r. data strongly supported the mechanism shown in Scheme 1a as the predominant (if not exclusive) mechanism of intermolecular hydrogen transfer in the lysine 2,3-aminomutase reaction. The participation of the alternative mechanism, Scheme 1b, appeared unlikely because this would have resulted in the formation of molecules having enriched 2-^{13}C, with deuterium at C-3. Thus, a β-shifted, but uncoupled, signal for C-2 would have been expected. However, it was conceivable that the shift might have been too small for resolution or the shifted signal might have been overlapped by the α-shifted triplet. Therefore we decided to carry out the complementary experiment in which a mixture of L-[3,3-$^2H_2$]-lysine plus L-[3-^{13}C]lysine was converted by the enzyme into β-lysine.

The required L-[3-^{13}C]lysine was synthesized by the route depicted in Scheme 2. The route parallels that used for our synthesis of L-[3,3-$^2H_2$]lysine.[1] A ^{13}C n.m.r. spectrum of the product showed, in addition to the expected enriched signal for C-3 (δ 31.10), a second enriched signal (*ca.* one-fourth the intensity of the δ 31.10 signal) at δ 42.70 due to enrichment at C-6. The latter may arise through some non-selectivity in the condensation of the methanesulphonate (**16**) with the sodium salt of diethyl phthalimidomalonate, although such lack of complete regioselectivity was not observed in our previous syntheses of [3,3-$^2H_2$]lysine using the same route.[2] Since the ^{13}C signal for C-6 of (**11**) (δ 37.30) is fairly well resolved (see Figure 1) from other signals, especially those of C-2 or C-3, the presence of some ^{13}C enrichment at C-6 was not considered to be a serious problem.

In order to enhance our chances of detecting any intermolecular exchange of deuterium at C-3, a mixture was prepared consisting of L-[3,3-$^2H_2$]lysine and L-[3-^{13}C]lysine (6:1). The mixture was converted by lysine 2,3-aminomutase into β-lysine, which allowed the reaction to proceed to equilibrium. Although equilibrium was reached in *ca.* 6 h, the incubation was continued for an additional 14 h. Thus it is quite likely that a substantial proportion of the substrate molecules was subjected to multiple turnover. The resultant β-lysine was isolated as (**11**), which was examined by ^{13}C n.m.r. spectroscopy. This spectrum showed a pair of enhanced singlets for C-3, shown in expanded form in Figure 2. The enhancements above natural abundance (as compared with the methoxy carbon signal, δ 51.75) are δ 47.61 (*ca.* 2.4 fold) and 47.65 (*ca.* 4.9 fold).* These correspond to 3-^{13}C enriched undeuteriated and

* The upfield signal due to enriched 3-^{13}C bearing deuterium will be substantially reduced in intensity due to the lack of the n.O.e. enhancement to which the downfield signal (due to enriched 3-^{13}C bearing protium) will have been subjected.



Scheme 2. Synthesis of L-[3-^{13}C]lysine. *Reagents:* i, $Na^{13}CN$–MeOH; ii, HCl–MeOH; iii, $LiAlH_4$; iv, methanesulphonyl chloride–Et_3N; v, sodium salt of diethyl phthalimidomalonate; vi, NaI–acetone; vii, potassium phthalimide; viii, HCl–HOAc–water; ix, *p*-nitrophenyl chloroacetate–NaOH; x, acylase I; xi, HCl–water

C-2 monodeuteriated (**11**), respectively. Thus, the majority of the enriched ^{13}C resonance due to C-3 had been subjected to an upfield β-shift, consistent with the mechanism outlined in Scheme 1a. The spectrum also showed for the C-2 carbon atom a singlet (δ 36.61), flanked by a 1:1:1 3-line signal centred at δ 36.25 (J = 20 Hz), the latter due to ^{13}C at natural abundance bearing a single deuterium atom. In addition, there appears an enhanced (*ca.* 2.8 times natural abundance) singlet at δ 37.34 resulting from the C-6 ^{13}C-enrichment of the precursor. However, no enriched triplet for C-3 directly bonded to deuterium could be seen, thus providing strong evidence against the intermolecular exchange of deuterium at C-3 as in Scheme 1b.

Discussion

In principle, at least two approaches are possible to distinguish between the mechanisms shown in Schemes 1a and 1b. The use of stereospecifically deuteriated (3*R*-$^2H_1$ and 3*S*-$^2H_1$) α-lysines in crossover experiments with either of the previously used heavier acceptor lysines (4,4,5,5-$^2H_4$ or U-^{13}C) would have allowed a distinction between the mechanistic possibilities, since it was known from our earlier work[2,3] that the 3-*pro-R* hydrogen is stereospecifically transferred to C-2 of β-lysine.

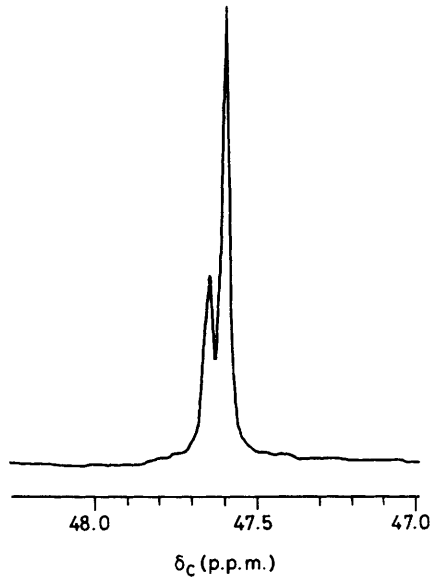


Figure 2. Expanded portion of the 125.76 MHz ^{13}C n.m.r. spectrum ($CDCl_3$ solution) of β-lysine di-*N*-phthaloyl methyl ester derivative (**11**) obtained after incubation of a mixture of L-[3,3-$^2H_2$]lysine + L-[3-^{13}C]lysine with lysine 2,3-aminomutase. The figure shows the unshifted (δ 47.65) and upfield β-shifted (δ 47.61) signals for C-3. *Conditions*: sweep width 29 411 Hz, repetition rate 0.557 s, relaxation delay zero, peak width 4.0 μs, line broadening 2 Hz, 1.795 Hz/pt; 11 402 scans

Thus, in Scheme 1a, intermolecular deuterium transfer would be expected from (3*R*)-[3-$^2H_1$]lysine and not from (3*S*)-[3-$^2H_1$]lysine, whereas in Scheme 1b, the expected pattern would be reversed. Unfortunately our supplies of (3*R*)-[3-$^2H_1$] and (3*S*)-[3-$^2H_1$]lysine were exhausted. The syntheses, even to the (2*RS*) compounds, which had not been resolved in our previous work,[2,3] were quite tedious, gave low yields, and were apparently not completely stereospecific.

We therefore chose an alternative approach to this problem, involving the crossover of deuterium from L-[3,3-$^2H_2$]lysine to specifically ^{13}C-enriched lysines. The location of intermolecularly transferred deuterium could be detected by its effects on the ^{13}C n.m.r. spectrum of the ^{13}C-enriched acceptor molecules. Thus, transformation of a mixture of L-[3,3-$^2H_2$]lysine plus L-[2-^{13}C]lysine gave β-lysine showing in the ^{13}C n.m.r. spectrum a deuterium-coupled enriched carbon as required by the mechanism of Scheme 1a, but lacking a β-shifted uncoupled enriched carbon as would be required by the mechanism of Scheme 1b. Similarly, transformation of a mixture of L-[3,3-$^2H_2$]lysine plus L-[3-^{13}C]lysine gave β-lysine showing an upfield β-shifted enriched signal for C-3, but no directly coupled enriched signal for C-3. The results strongly support our earlier conclusion[1] that the migrating 3-*pro-R* hydrogen of α-lysine is transferred to C-2 essentially completely intermolecularly. No intermolecular exchange of the 3-*pro-S* hydrogen (deuterium) of α-lysine remaining at C-3 could be detected.

As discussed in our earlier paper,[1] the intermolecular nature of this hydrogen transfer suggests the involvement of some hydrogen transferring group (designated X in Scheme 1). The enzyme does not use AdoCbl as coenzyme, and the involvement of C-5′ of the required coenzyme *S*-adenosylmethionine has been ruled out.[1,*] For reasons discussed in the literature[8]

* Evidence conflicting with this conclusion has recently been published: M. Moss and P. A. Frey, *J. Biol. Chem.*, 1988, **262**, 14859.

with regard to AdoCbl-dependent intermolecular hydrogen transfer processes, this 'X' group must have at least one hydrogen to account for the return to a particular substrate molecule of a hydrogen atom not originally derived from that same molecule. Thus, for example, aspartate or glutamate carboxylate anions or histidine could not serve in this role, but the side chain amino groups of lysine or arginine residues in the active site of lysine 2,3-aminomutase might, in theory, be acceptable candidates. Further studies on this rearrangement are in progress and will be reported in due course.

Experimental

For general experimental details, see ref. 1. ^{13}C N.m.r. spectra of β-lysine derivatives (**11**) were taken on a Bruker WM-500 instrument. ^{13}C N.m.r. spectra of labelled lysines and their synthetic precursors were taken on a Bruker WM-250 instrument. L-[2-^{13}C]Lysine monohydrochloride was obtained from Merck. A ^{13}C n.m.r. spectrum (D_2O + 0.10M KPO_4; pH 7.0 with dioxane, δ 67.8, as the internal reference) confirmed that all of the labelling was located at C-2 (δ 55.75). (Unlabelled lysine, in this solvent, gave peaks at δ 22.72, 27.61, 31.12, 42.34, 55.69, and 175.90.)

Lysine 2,3-aminomutase was purified from *Clostridium subterminale* strain SB4 (ATCC 29748) as previously described.[1] For incubations with mixtures of L-[3-^{13}C]lysine plus L-[3,3-$^{2}H_2$]lysine, freshly prepared enzyme was used. For incubations with mixtures of L-[2-^{13}C] lysine plus L-[3,3-$^{2}H_2$] lysine, enzyme was used which had been prepared *ca.* six months earlier. When prepared, the enzyme had been frozen using solid CO_2–acetone in *ca.* 1 ml aliquots (*ca.* 1.0 unit[1] of aminomutase activity; *ca.* 5 mg protein) in ampoules under N_2, and stored at −70 °C. After having been thawed, the activity of the enzyme was apparently unchanged.

Enzyme assays were performed, and incubations followed, by using a new h.p.l.c. technique, reported elsewhere.[4] Conversions of mixtures of substrates (25 mg each component) and conversions of the products into the di-*N*-phthaloyl methyl ester derivatives (**10**) and (**11**) were carried out as previously described.[1]

Synthesis of L-[3-^{13}C]*Lysine* (**20**).—A mixture of 1-chloro-3-iodopropane (**12**) (12.24 g, 59.87 mmol) and sodium [^{13}C]-cyanide [5 g, 100 mmol, 99 atom% ^{13}C] in MeOH (120 ml) was refluxed for 5 h. The MeOH was evaporated under reduced pressure, and the residue was mixed with ether and water. The ether extract was washed (saturated NaCl), dried (Na_2SO_4), and evaporated to yield a yellow oil (4.9 g) which was distilled in a short path apparatus to yield [1-^{13}C]-4-chlorobutyronitrile (**13**) (4.5 g), b.p. 90—100 °C (15 mmHg), δ($CDCl_3$) 14.74, 28.22, 42.80, and 118.67 (enhanced intensity).

The above product (4.4 g) was dissolved in MeOH (45 ml) and the solution was saturated with dry HCl (without cooling). The solution was refluxed for 16 h after which time it was concentrated under reduced pressure to 15 ml, diluted with water, and extracted with ether. The extract was washed (water, diluted aqueous $NaHCO_3$, saturated aqueous NaCl), dried (Na_2SO_4), and evaporated to yield an oil. This was distilled at atmospheric pressure through a short path apparatus to yield methyl [1-^{13}C]-4-chlorobutyrate (**14**), (3.7 g), b.p. 170—175 °C, δ($CDCl_3$) 27.66, 30.92, 43.99, 51.57, and 172.96 (enhanced intensity).

The above product was added dropwise over 15 min to a suspension of $LiAlH_4$ (0.8 g) in ether (40 ml) at −10 to −15 °C, with mechanical stirring, under N_2. Stirring was continued for 10 min at −10 °C, and then the mixture was allowed to warm to room temperature and stirring was continued for a further 30 min. The mixture was cooled to 0 °C and saturated aqueous Na_2SO_4 (4 ml) was added dropwise over 15 min. The mixture was vacuum filtered and the residue was washed with ether. The filtrate was dried (Na_2SO_4) and evaporated to yield an oil which was distilled in a short path apparatus to yield [1-^{13}C]-4-chlorobutan-1-ol (**15**) (2.28 g), b.p. 78—80 °C (15 mmHg), δ($CDCl_3$) 29.11, 29.80, 44.93, and 61.81 (enhanced intensity).

The product (2.18 g, 20.1 mmol) was dissolved in CH_2Cl_2 (15 ml). The solution was cooled to −78 °C and treated with methanesulphonyl chloride (2.37 g, 20.1 mmol). The mixture was recooled to −78 °C, and triethylamine (2.1 g, 20.8 mmol, freshly distilled) was added dropwise over 10 min, to give a white precipitate. The mixture was stirred at −78 °C for 15 min, then allowed to warm to room temperature and kept at this temperature for 30 min. The mixture was then poured into water (50 ml) and the CH_2Cl_2 phase separated. The aqueous phase was washed with additional CH_2Cl_2. The combined CH_2Cl_2 extracts were dried (Na_2SO_4) and concentrated to yield an oil which was distilled in a short path apparatus to give [1-^{13}C]-4-chlorobutyl methanesulphonate (**16**) (1.74 g), b.p. 115—120 °C (0.5 mmHg). The latter was converted, as previously described for [1,1-$^{2}H_2$]-4-chlorobutyl methanesulphonate,[1] without isolation of intermediates, into (2*RS*)-[3-^{13}C]lysine (**19**) (0.86 g).

The product was resolved by a published procedure[9] to yield L-[3-^{13}C]lysine monohydrochloride (**20**) (130 mg), δ(D_2O + 0.1M KPO_4; pH 7.0; internal dioxane δ 67.8) 31.10 (relative intensity 4.5) and 42.70 (relative intensity 1.0).* Analysis of the product using Marfey's reagent[10] as previously described[1] showed no trace of D-lysine.

Acknowledgements

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* This spectrum was taken on a very small sample, and only the enriched carbons were detected.

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